ARTICLES

Endotoxin and immune activation in chronic heart failure: a prospective cohort study

Josef Niebauer, Hans-Dieter Volk, Michael Kemp, Martin Dominguez, Ralf R Schumann, Mathias Rauchhaus, Philip A Poole-Wilson, Andrew J S Coats, Stefan D Anker

Summary

Background Immune activation in patients with chronic heart failure may be secondary to endotoxin (lipopolysaccharide) action. We investigated the hypothesis that altered gut permeability with bacterial translocation and endotoxaemia would be increased in patients with oedema secondary to congestive heart failure.

Methods We compared 20 patients who had chronic heart failure with recent-onset peripheral oedema (mean age 64 years [SD 10], New York Heart Association [NYHA] class 3·3 [0-7]), 20 stable non-oedematous patients with chronic heart failure (mean age 63 years [19], NYHA class 2·6 [0-7]), and 14 healthy volunteers (mean age 55 years [16]). Biochemical markers of endotoxaemia, inflammation, and immune activation were measured. Ten patients were studied within 1 week of complete resolution of oedema. Five patients survived longer than 6 months and were restudied again after remaining free of oedema for more than 3 months.

Findings Mean endotoxin concentrations were higher in oedematous patients with chronic heart failure than in stable patients with chronic heart failure (0·74 [SD 0·45] *vs* 0·37 EU/mL [0·23], p=0·0009) and controls (0·46 EU/mL [0·21], p=0·02). Oedematous patients had the highest concentrations of several cytokines. After short-term diuretic treatment, endotoxin concentrations decreased from 0·84 EU/mL [0·49] to 0·45 EU/mL [0·21], p<0·05) but cytokines remained raised. After freedom of oedema for more than 3 months after oedema resolved, endotoxin concentrations remained unchanged from the previous visit (0·49 EU/mL [0·06], p=0·45).

Interpretation Raised concentrations of endotoxin and cytokines are found in patients with chronic heart failure during acute oedematous exacerbation. Intensified diuretic treatment can normalise endotoxin concentrations. Our preliminary findings suggest that endotoxin may trigger immune activation in patients with chronic heart failure during oedematous episodes.

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Cardiac Medicine, National Heart and Lung Institute, Imperial College School of Medicine, London, UK (J Niebauer MD, M Kemp MRCP, M Rauchhaus, Prof P A Poole-Wilson MD, Prof A J S Coats DM, S D Anker MD); Kardiologie, Herzzentrum der Universität Leipzig, Germany (J Niebauer); Institut für Medizinische Immunologie, Universitätsklinikum Charité, Berlin, Germany (Prof H-D Volk MD); Heart Science Center, Harefield Hospital, UK (M Dominguez MSc); Institut für Mikrobiologie und Hygiene, Universitätsklinikum Charité, Berlin (R R Schumann MD); and Franz-Volhard-Klinik am Max-Delbrück Centrum für Molekulare Medizin, Charité, Campus Berlin-Buch, Berlin (S D Anker)

Correspondence to: Dr Stefan Anker, Cardiac Medicine, National Heart and Lung Institute, London SW3 6LY, UK (e-mail: s.anker@ic.ac.uk)

Introduction

Some patients with chronic heart failure have features such as cardiac cachexia that may be due to activation of the immune system.[1,2] Increased expression of tumour necrosis factor α (TNFα) has been found in cardiac tissue of patients with chronic heart failure undergoing heart transplantation and the failing heart has been suggested as the cause of immune activation.[3] No link between a pathogenic process and cytokine activation has been documented in human beings with heart failure or in animal models. The cause of increased cytokine production in patients with heart failure remains unknown.

We have previously suggested that bacterial endotoxin, lipopolysaccharide, contributes to immune activation in chronic heart failure.[4] Acute venous congestion could lead to altered gut permeability for bacteria, endotoxin, or both, and to translocation of these materials into the circulation. In the circulation, lipopolysaccharide is bound by a serum protein, termed lipopolysaccharide-binding protein (LBP).[5] The lipopolysaccharide-LBP complex can interact with the CD14 membrane protein and Toll-like signalling receptors to start a signalling cascade that leads to increased cytokine production (figure 1). The extracellular domain of the CD14 receptor is shed after interaction and serum concentrations are thought to reflect the amount of endotoxin and cell interaction. The lipopolysaccharide-LBP ratio has been shown to be crucial for the immunostimulatory effects of lipopolysaccharide.[6] High concentrations of LBP, as seen during the acute-phase response, can completely block lipopolysaccharide effects in vitro and in a murine sepsis model.[7] Furthermore, patients with high concentrations of soluble CD14 (which shows endotoxin-cell interaction and shedding of CD14 from the cell membrane[8]) have strikingly increased concentrations of TNFα, soluble TNF receptor-1 and receptor-2, and intracellular-adhesion molecule-1.[4]

The degree of bowel-wall oedema cannot be directly measured. The relation between central haemodynamics and the pathophysiological features of chronic heart failure is weak.[9] In animal models there is a poor relation between intracardiac pressures and intestinal perfusion.[10] We therefore separated patients according to the presence or absence of a reliable marker of acute venous congestion due to cardiac failure, namely peripheral oedema. Bowel-wall oedema that could cause altered gut permeability and bacterial (ie, endotoxin) translocation is most likely to occur with moderate to severe peripheral oedema.

Our main aim in this study was to measure endotoxin and cytokine concentrations in patients with chronic heart failure during an acute exacerbation with peripheral oedema and after short-term and long-term treatment with diuretics.

Methods

Participants

We studied prospectively 14 healthy volunteers (mean age 55 years [SD 16]) and 40 patients with chronic heart failure (mean age 68 years [15], p=0·11). We did the baseline studies between April and October, 1997. 20 stable patients were recruited during outpatient clinics on 3 specific days and 20 patients with moderate

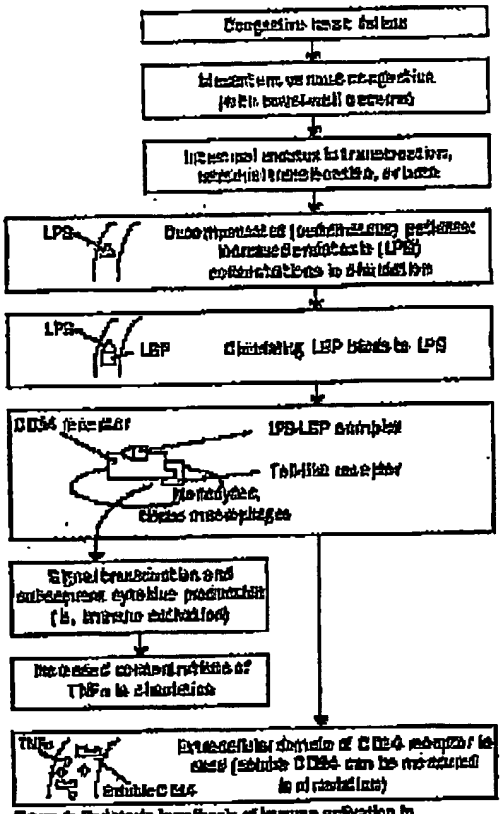

Figure 1: Endotoxin hypothesis of immune activation in congestive heart failure
LPS=lipopolysaccharide.

or severe oedema represented all decompensated patients at Royal Brompton Hospital, London, UK, during the time period, identified on attendance to the clinic or admission to the ward. Healthy volunteers were hospital staff and relatives of patients who agreed to participate. Only one healthy person declined participation. We excluded data from three volunteers aged younger than 35 years to achieve a similar mean age in all groups. The causes of chronic heart failure were ischaemic in 27 patients and idiopathic dilated cardiomyopathy in 13 patients. The diagnosis of chronic heart failure was based on symptoms arising during exercise, cardiomegaly, and documented left-ventricular dysfunction (all patients had a left-ventricular ejection fraction measured by echocardiography or radionuclide ventriculography of <40%). No patient or volunteer had clinical signs of infection, rheumatoid arthritis, or cancer.

Patients were treated with diuretics (n=38), angiotensin-converting-enzyme inhibitor (n=36), digoxin (n=14), aspirin (n=17), amiodarone (n=16), and nitrates (n=15) in various combinations. We did extended follow-up of ten oedematous patients who lived close to our hospital (five New York Heart Association (NYHA) class IV, five class III) after treatment with increased doses of diuretics (furosemide up to 120 mg/day, with addition of bendrofluazide 2·5 mg or 5·0 mg once daily, metolazone 5 mg or 10 mg once daily, or both). Of these patients, three had to be admitted for 5–8 days for intravenous diuretic treatment.

After a median of 14 days (range 7–89) we remodied these patients within 1 week of complete resolution of oedema (after treatment six NYHA class III, four class II; mean weight loss 3·6 kg [0·5, range 2·5–5·0]). Five patients regained clinical stability (one NYHA class III, four class II) and were remodied again 14–52 weeks (mean 31 weeks [7]) after the initial investigation when they had been free of peripheral oedema for more than 3 months. The remaining five patients did not remain a stable clinical state and died 2–8 months after the initial investigation without having been remodied. The research protocol was approved by the ethics committee of the Royal Brompton Hospital, and all patients and volunteers gave written informed consent.

Assays

Blood samples were collected after rest for at least 15 min. A polyethylene catheter was inserted into an antecubital vein and 8 mL of blood were drawn into endotoxin-free tubes (Endo Tube HT, Chromogenix AB, Sweden). 30 mL samples were also taken for biochemical and cytokine measurements. After immediate centrifugation, blood and plasma samples were stored at −80°C until analysis. In addition, 5 mL blood was taken into tubes containing edetic acid for fluorescence-activated cell-sorting analysis.

Concentrations of endotoxin were measured with a commercially available kit (Limulus Amebocyte Lysate QCL-1000 test kit, BioWhittaker Inc, Walkersville, USA). The normal concentration of endotoxin in this assay in healthy people is <0·50 EU/mL. The within-assay coefficients of variation at concentrations of 0·35 EU/mL and 0·82 EU/mL were 9·9% and 9·6%; between-assay coefficients of variation were 16·8% and 13·3%, respectively. For repeated blood samples in non-oedematous patients the coefficient of variation was 10·8%. The lower limit of detection was 0·03 EU/mL.

LBP was measured by ELISA.[8] Total TNFα was measured with an ELISA kit (Medgenix, Fleurus, Belgium; sensitivity 3·0 pg/mL; test not influenced by soluble TNF receptors). ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure soluble TNF receptor-1 and receptor-2, and interleukin 6; lower limits of detection of the assays were 25 pg/mL, 2 pg/mL, and 0·0094 pg/mL, respectively. Soluble CD14 was assessed by ELISA (IBL, Hamburg, Germany). Plasma procalcitonin concentrations were measured by an immunoluminometric assay (BRAHMS, Berlin, Germany).[9]

In a subgroup of ten non-oedematous and seven oedematous patients, as well as in all healthy volunteers, whole-blood samples were taken in potassium edetic-acid tubes (Vacutainer Systems, Falcon BD, Oxford, UK) and stained with fluorescently labelled monoclonal antibodies (Coulter Electronics, Luton, UK) to

	Healthy volunteers (n=24)	CHF no oedema (n=20)	CHF oedema (n=20)
Demography (mean [SD])			
Age (years)	58 (15)	63 (15)	64 (10)
Weight (kg)	74 (7)	76 (9)	78 (9)
NYHA class	..	2·6 (0·7)	3·3 (0·5)*
Cause			
Ischaemic	..	15	11
Idiopathic dilated	..	4	9
Clinical (mean [SD])			
Sodium (mmol/L)	139 (1·5)	137 (4·5)	134 (4·8)†
Creatinine (μmol/L)	82 (15)	131 (58)	219 (160)†§
Urea (mmol/L)	5·4 (0·5)	11·6 (7·6)	20·0 (12·6)†§
Uric acid (μmol/L)	308 (83)	417 (146)‖	640 (174)*‡
Aspartate aminotransferase (U/L)	26 (10)	24 (8)	23 (8)
Alanine aminotransferase (U/L)	23 (10)	17 (4)†	14 (4)‖
Lymphocyte profile (mean [SD])			
CD4	47 (8)	51 (11)	35 (13)‖¶
CD8	22 (7)	25 (14)	28 (20)
CD4/8 ratio	2·5 (1·2)	2·3 (2·4)	2·6 (2·3)
CD4/25 ratio	6·7 (4·2)	5·5 (2·4)	10·6 (9·7)
CD8/25 ratio	4·7 (2·4)	8·7 (4·6)	22·6 (10·7)‖

CHF=chronic heart failure.
*p<0·001 vs CHF no oedema. †p<0·01 vs healthy volunteers. ‡p<0·001 vs healthy volunteers. §p<0·05 vs CHF no oedema. ‖p<0·05 vs healthy volunteers. ¶p<0·01 vs CHF no oedema.

Table 1: Characteristics of patients with chronic heart failure with and without peripheral oedema and healthy volunteers

	Healthy volunteers (n=14)	CHF no oedema (n=20)	CHF oedema (n=20)
Endotoxin (EU/mL)	0·48 (0·21)	0·37 (0·23)	0·74 (0·45)*†
LBP (µg/mL)	8·8 (4·6)	10·4 (5·3)	12·1 (6·0)
Lipopolysaccharide/log LBP ratio	0·54 (0·20)	0·44 (0·30)	0·75 (0·48)‡
TNFα (pg/mL)	24·8 (9·6)	95·8 (7·9)	35·6 (12·3)‡§
Soluble TNF receptor-1 (pg/mL)	708 (218)	1077 (529)	1822 (1396)‡‖
Soluble TNF receptor-2 (pg/mL)	1465 (335)	2088 (1380)	3143 (1690)‡¶
Soluble CD14 (ng/mL)	3456 (563)	3514 (464)	4243 (586)‡‖
Procalcitonin (µg/mL)	87 (1·5)	109 (73)	145 (94)
Interleukin-6 (pg/mL)	2·0 (0·4)	4·3 (5·5)	14·7 (17·3)‡§
C-reactive protein (mg/L)	5·6 (1·7)	8·5 (9·5)	19·7 (17·1)‡¶

*p<0·05 vs healthy volunteers. †p<0·001 vs CHF no oedema. ‡p<0·01 vs CHF no oedema. §p<0·01 vs healthy volunteers. ‖p<0·001 vs healthy volunteers. ¶p<0·05 vs CHF no oedema.

Table 2: Mean (SD) plasma concentrations of endotoxin and inflammatory markers in healthy volunteers and patients with chronic heart failure

determine peripheral lymphocyte phenotype and the proportion of CD25 receptor-positive T cells. A staining excess of antibody, determined by titration (data not shown), was placed into 12×75 mm polypropylene tubes (Kiloy, Hampshire, UK). Two tubes were analysed for each patient's sample. The first tube contained control monoclonal mouse antihuman antibodies isotypically matched to the test antibodies in the second tube. The antibody-fluorochrome conjugates used were CD3-PC5, CD4-FITC, CD8-ECD, and CD25R-RD1. The formic-acid lysed whole-blood protocol was used in the multi-Q-prep (Coulter Electronics, Luton, UK). Lymphocyte gating was set on forward compared with side-scatter dot plot, and compensation was established by the combining of single-colour-stained leucocyte populations. Four-colour flow cytometry was done on the Coulter XL-MCL with System II software (version 2.0).

Statistical analysis

We assessed normality of distribution with the Kolmogorov-Smirnov test. Unpaired Student's *t* test, paired *t* test, ANOVA with Fisher's post-hoc test (with allowance for multiple testing), and the Mann-Whitney *U* test were used where appropriate. Data are presented as mean (SD). We also used univariate-correlation and multivariate-correlation analyses to establish the relation between variables. We took $p<0·05$ to be significant.

Results

In tables 1 and 2, baseline clinical characteristics and results of immunological and humoral measurements are shown. Endotoxin concentrations were highest in heart-failure patients with peripheral oedema, compared with heart-failure patients without oedema (98%) and controls (59%, p=0·0027; figure 2). Plasma concentrations of LBP did not differ between groups, but there was a raised lipopolysaccharide/log LBP ratio in the heart-failure patients with oedema compared with those without oedema (71%, p<0·01). In oedematous heart-failure patients, plasma concentrations were significantly higher for C-reactive protein, TNFα, soluble TNF receptor-1 and receptor-2, interleukin-6, and soluble CD14 (table 2) than for all other groups.

Among all participants (n=54), concentrations of soluble CD14 correlated significantly with endotoxin ($r=0·30$, $p<0·05$). This correlation was not significant when patients or healthy volunteers were analysed separately. In all patients with chronic heart failure, soluble CD14 correlated with TNFα ($r=0·32$, $p<0·05$) and soluble TNF receptor-1 ($r=0·45$, $p<0·01$). There was a correlation between soluble CD14 and soluble TNF receptor-2 in patients with stable chronic heart failure ($r=0·61$, $p<0·01$).

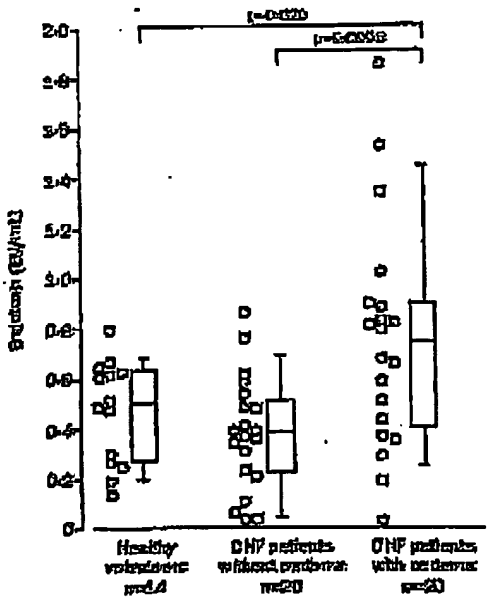

Figure 2: Plasma endotoxin concentrations in healthy volunteers and heart-failure patients with and without oedema
Short horizontal lines=10th and 90th percentiles; long horizontal lines=25th, 50th, and 75th percentiles.

No simple correlations existed between creatinine or urea plasma concentrations and lipopolysaccharides at baseline, nor between changes in markers of kidney function over time compared with changes of lipopolysaccharide or cytokine concentrations over time (data not shown). Therefore, a bias because of latent abnormalities of kidney function seen in some oedematous patients is unlikely.

Intensive diuretic treatment for a mean of 23 days (8) in ten patients with chronic heart failure resulted in a mean weight decrease of 3·6 kg (range 2·5–5·0), and improvement in the functional NYHA class in nine of the ten patients. In eight of these, endotoxin plasma concentration was decreased from 0·96 EU/mL (0·47) to 0·45 EU/mL (0·24). In two patients with normal concentrations of endotoxin at baseline, concentrations after diuretic treatment were 9% and 36% higher than at baseline, but still in the normal range (<0·5 EU/mL). In all ten patients the lipopolysaccharide concentrations fell from 0·84 EU/mL (0·49) to 0·45 EU/mL (0·21, p=0·049;

	Baseline (n=10)	After diuretic treatment (n=10)	p
Endotoxin (EU/mL)	0·84 (0·49)	0·45 (0·21)	<0·05
LBP (µg/mL)	10·3 (5·7)	12·7 (7·6)	0·27
Lipopolysaccharide/log LBP ratio	0·85 (0·44)	0·28 (0·67)	0·033
TNFα (pg/mL)	33·4 (19·2)	40·2 (13·6)	0·62
Soluble TNF receptor-1 (pg/mL)	2138 (1314)	2785 (1391)	0·09
Soluble TNF receptor-2 (pg/mL)	3791 (1185)	4029 (1497)	0·46
Soluble CD14 (ng/mL)	4474 (537)	4430 (764)	0·89
Procalcitonin (ng/mL)	153 (92)	219 (104)	0·17
Interleukin-6 (pg/mL)	19·4 (23·0)	18·3 (23·6)	0·69
C-reactive protein (mg/L)	19·6 (21·0)	20·0 (20·7)	0·00

Table 3: Mean (SD) plasma concentrations of endotoxin and inflammatory markers in oedematous patients before and after diuretic treatment

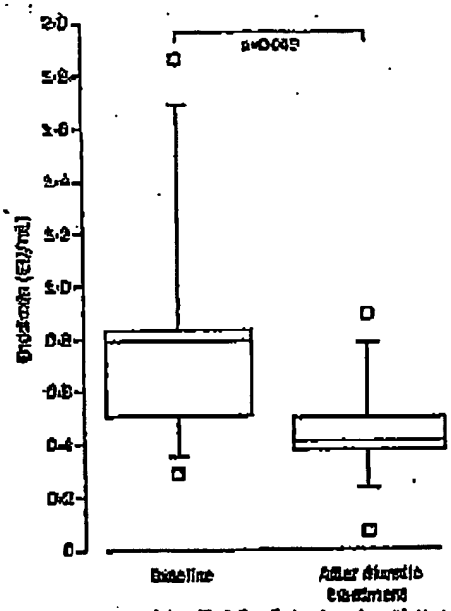

Figure 3: Effect of intensified diuretic treatment on plasma endotoxin concentrations in ten patients with chronic heart failure who had peripheral oedema
Short horizontal lines=10th and 90th percentiles; long horizontal lines=25th, 50th, and 75th percentiles; o=duration value outside 10th and 90th percentiles.

figure 3). The effect of diuretic treatment on the endotoxin and inflammatory markers are shown in table 3. During extended follow-up, five patients were remodled when free of oedema for more than 3 months after 21 weeks (7). Endotoxin concentrations at the third visit did not differ from those at the second visit after a mean of 19 days (0·39 [0·22] *vs* 0·49 EU/mL [0·06], p=0·45), but TNFα concentrations were lower (39·6 [12·4] *vs* 31·0 pg/mL [5·7], p=0·079)

Discussion

We have shown that endotoxin concentrations and proinflammatory cytokines are raised in patients with heart failure who have peripheral oedema. Raised endotoxin concentrations were normalised by heightened diuretic treatment. The endotoxaemia in these patients was not associated with a strong acute-phase response that would have led to an increased hepatic LBP synthesis and subsequent blocking of lipopolysaccharide effects. These results lend credence to the hypothesis that bacterial endotoxin may be an important stimulus of immune activation in patients with chronic heart failure. This finding may open various options for treatment of patients with chronic heart failure that could be directed against bacteria in the bowel, the translocation process, and endotoxin itself, the binding sites of bacterial endotoxin on immune competent cells, or both.

The complex of endotoxin and endotoxin-binding protein activates monocytes and tissue macrophages via the CD14 and Toll-like receptor proteins,[8] which stimulates the production of TNFα and other cytokines (figure 1). Previous studies suggested that increased soluble CD14 concentrations might be related to endotoxaemia.[9] We established that oedematous patients have the highest concentrations of soluble CD14 and lipopolysaccharide, but in homogeneous groups of patients there was no direct numeric relation between the two variables. Shed, and therefore soluble, CD14 receptors are thought to reflect the amount of endotoxin/cell interaction in the long term. By contrast, endotoxin has a short plasma half-life (10–30 min), which may explain why soluble CD14 concentrations are more closely related to cytokine than endotoxin concentrations.[4]

The concentrations of endotoxin in our study were well below those seen in septic shock.[10] Patients with chronic heart failure had no signs of active infection, and the moderate increases in plasma endotoxin are in keeping with the hypothesis of a translocation process. Possibly, it is endotoxin itself rather than bacteria that translocates. Lipopolysaccharides at baseline did not correlate significantly with renal function (as estimated by creatinine and urea) although this finding cannot completely exclude an influence of renal function on cytokine clearance.

Although intensified diuretic therapy resulted in normalisation of endotoxin concentrations, treatment did not lead immediately to lowered cytokine plasma concentrations, which is in keeping with a previous study.[15] This effect may be due to a concentration effect, resulting from the loss of up to 5 kg body water or long-term activation of monocytes or macrophages after brief exposure to an endotoxin stimulus during a phase of clinical deterioration with increased venous congestion. Alternatively, the lack of cytokine decrease immediately after clinical improvement may be due to a change in monocyte or macrophage lipopolysaccharide sensitivity (ie, "normalised" endotoxin concentrations may still cause increased cytokine production). Indeed, such an increased cellular sensitivity to lipopolysaccharides has been documented in patients with chronic heart failure who had acute decompensation.[11] The previously documented raised TNFα concentrations in cardiac tissue of patients with endstage chronic heart failure may also be due to cardiomyocytes or tissue monocytes releasing increased amounts of cytokines upon stimulation by lipopolysaccharides because of decompensation or hypersensitive cardiomyocytes. In cardiomyocytes of heart transplantation recipients (especially in patients with ischaemic chronic heart failure) increased baseline and lipopolysaccharide-stimulated TNFα production has been reported.[11] In our study, after a long phase of clinical stability, TNFα plasma concentrations showed a strong trend to decrease back to normal, and, therefore, the process of normalisation of cytokine secretion seems to be slow.

Tolerance of monocytes or macrophages to endotoxin can be induced in vivo and in vitro by endotoxin itself. Such an effect frequently occurs after severe injury.[12] One important mediator of lipopolysaccharides hyposensitivity is interleukin-10.[13] Compared with controls, we found interleukin-10 to be lower in stable patients with chronic heart failure.[9] Increased cardiac-wall stress and general tissue hypoxia (both via local free-radical generation and subsequent stimulation of the nuclear factor-κ B pathway) and hormonal catabolic and anabolic imbalance (especially in patients with muscle wasting[14]) may lead to immunological hypersensitivity. Endotoxin may, therefore, be an important stimulus for cytokine production in the heart and in the periphery even in the absence of oedema. In-vitro low concentrations of lipopolysaccharides have

detrimental effects on cardiomyocytes. These effects are indirect through the release of other substances,[21] but direct effects also have been seen.[22] In vivo there may be a dynamic balance between heart function and immune activation in patients with chronic heart failure, and over time patients with frequent oedematous episodes may deteriorate because of the cardiodepressant and metabolic consequences of raised TNFα concentrations. Better control of oedema in chronic heart failure may therefore, be beneficial.

In stable ambulatory patients with chronic heart failure, a significant excess concentration of cytokines from the heart could not be shown,[23] which suggests that cardiac production may not be the main source of the raised peripheral cytokine plasma concentrations. In support of the importance of peripheral hypoxia, measures of increased oxidative stress have been found to correlate with soluble TNF receptor-1 and receptor-2 concentrations.[24] We have shown that peak leg blood flow after ischaemia in clinically stable patients with chronic heart failure is inversely related to TNFα plasma concentrations. This effect may be due to a relation between hypoxia and TNFα production or toxic effects of TNFα on endothelial function.[25] Hypoxia may not be the most important cytokine trigger in chronic heart failure because of the cytokine profile. Raised interleukin-6 plasma concentrations can be attributed to peripheral hypoxic conditions,[26] which will occur in chronic heart failure, but there is no report that hypoxia leads to raised concentrations of TNFα, procalcitonin, or soluble TNF receptor-1 or receptor-2. Soluble CD14 receptors are, by contrast, characteristic of endotoxin action, but not of hypoxic disorders.[27]

This study shows the presence of raised plasma endotoxin concentrations in patients with chronic heart failure and peripheral oedema. In the presence of unchanged concentrations of endotoxin binding protein, the raised endotoxin concentration reflects a potentially pathogenic situation that leads to cytokine induction. We show that normalisation of endotoxin concentrations can be achieved by intensified diuretic treatment. Bacterial endotoxin may be an important stimulus of immune activation in patients with chronic heart failure. Our studies are preliminary and further investigations are needed. Nevertheless, these findings may open various new options for treatment directed against bacteria in the bowel, the translocation process, and endotoxin itself, the binding sites of bacterial endotoxin on immune competent cells, or both.

Contributors

Stefan Anker and Andrew Coats developed the endotoxin hypothesis and with Philip Poole-Wilson designed the study. Josef Niebauer and Stefan Anker coordinated the study. Josef Niebauer did all clinical assessments with the help of Mathias Rauchhaus. Michel Kemp measured endotoxin and TNFα. Martin Dominguez did all lymphocyte analyses. Hans-Dieter Volk and Ralf Schumann advised on immunological issues and measured all other cytokines and LBP. Josef Niebauer and Stefan Anker analysed the data and prepared the manuscript with the help of Philip Poole-Wilson and Andrew Coats. Hans-Dieter Volk and Ralf Schumann edited the manuscript.

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